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DESIGN OF MAXIMALLY IMMUNOGENIC VACCINES FROM SYNTHETIC PEPTIDES LINKED TO POLYMER CARRIERS

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19. Abstract: Considerable and encouraging progress has been made in fulfilling the aims set forth in our original research proposal. Using relevant peptides from the circumsporozoite coat protein of P.falciparum and P.bergei, we have successfully constructed peptide-polymer conjugates of optimal immunogenicity in mice. We chose dextran as the carrier polymer of choice after experimenting with a number of carriers of different chemical and physical characteristics. The molecular parameters crucial for the construction of the immunogen were determined. These involved using a dextran carrier molecule of optimum molecular mass coupled to peptide in such a way as to yield an optimum peptide density and valence per molecule. Importantly, as we originally proposed, these conjugates were immunogenic without the use of adjuvants. The constructed antigenic conjugates were essentially of the T-cell Independent type, and gave rise primarily to antibody of the IgM isotype.

Building on the information gained from the determination of the optimal molecular characteristics necessary for peptide-dextran conjugates to stimulate IgM responses, we next concentrated on stimulating IgG antibody responses. This was done because protective, *neutralizing antibodies are often IgG isotypes of higher affinity. We synthesized* the polyproteins poly-BSA and poly-OVA for use as carriers. By themselves, these polyproteins induced IgG as well as IgM responses and immunogenicity increased with increasing multiplicity of protein. Injection protocols were optimized so as to yield maximal immunogenicity using the lowest doses feasible. As with peptide-dextran conjugates, polyproteins were immunogenic without adjuvant use, and the resultant immune response approached in magnitude the level of antibody produced by injecting polyproteins in Freund's adjuvant.

Making use of the above information as to the optimal protocols and doses to be used with poly-proteins, preliminary model experiments have been carried out to explore the use of polyproteins as carriers for conjugation to relevant peptides. Preliminary experiments using the hapten, fluorescein, as a "model epitope" have been carried out. Fluoresceinated poly-BSA generated anti-fluorescein IgG responses, the response rising to a peak with increasing degree of substitution, and then falling rapidly with increasing substitution, indicating that there is an optimal epitope density beyond which response diminishes.

Design of Maximally Immunogenic Vaccines from Synthetic Peptides Linked to Polymer Carriers: Three Year Summary Report

In recent years, a new approach to the construction of vaccines has involved the isolation or synthesis of specific peptides from the outer surfaces of pathogenic or parasitic organisms for use in the vaccine. These peptides are important in that they have been selected as relevant immunogenic determinants of the molecules of which they are fragments. The ultimate aim of this approach is to use these relevant peptides to generate not only an immune response against the larger molecules to which they belong, but also to generate protective antibodies against the parent pathogen. Interest has focussed on those polypeptide regions in the surface proteins of a pathogen that have a constant and essential biological function and whose structure cannot vary. If such a region were to be incorporated into a vaccine and made to induce an immunogenic response, the resultant vaccine would be a "universal" one, in that it would protect against most variants of the particular pathogen.

Since peptides are not generally by themselves immunogenic, they are typically linked to a large "carrier" protein and then injected along with any one of a number of adjuvants. Adjuvants are substances which tend to increase the level and duration of immunity; however, they are often toxic and can cause irritation at the site of injection.

The aim of our three year project has been to develop a general method for constructing a vaccine containing a relevant peptide from a pathogen, linked to a carrier molecule of optimized chemistry and geometry. Because of the extensive experience of laboratories at the Walter Reed Army Institute of Medical Research with relevant peptides of malarial proteins, these were considered the peptides of choice for polymer conjugation. Experiments aimed at finding the ideal carrier molecule as well as the optimal spacing and number of peptides on that molecule were to be based on our laboratory's extensive experience with achieving maximal immunogenicity of haptens linked to carrier polymers. This general method of vaccine construction was to rely on generating optimal peptide-polymer conjugates of such reproducible and effective immunogenicity that the need for the use of adjuvants would be minimized or eliminated.

On the next several pages is the overview of the approach to this project during the three year grant period.

Year 1 Outline:
Molecular Parameter Optimizations of Polymer & Epitope

I. Polymer Suitability Studies:

A. Testing for appropriate polymers; properties considered: Natural? Synthetic? Cross-linked? Linear? Charged? Uncharged? Homopolymer vs Heteropolymer?

B. Testing for optimal molecular parameters necessary to enhance immunogenicity.

C. Testing for molecular parameters which might inhibit the immune response; avoidance of their synthesis and/or avoidance of their inclusion as contaminants in vaccine construction.

II. Haptens as Model "Epitopes" for Conjugation to Polymers

A. Conjugation of haptens to polymers.

B. What hapten valence per molecule is optimal for immunogenicity?

C. What hapten density per molecule is optimal for immunogenicity?

III. Preliminary Studies on Malarial Peptide-Polymer Conjugates

A. Synthesis of a relevant malarial peptide (triple-repeat of a tetra-peptide of the circumsporozoite protein of P.falciparum).

B. Protocol optimization for attaching a peptides to polymers.

C. Synthesis of a trial peptide-dextran conjugate.

D. In vivo immunogenicity in mice of a multivalent peptide-dextran conjugate.

Aim and Scope of Year 1 Studies:

The research in year 1 centered around a search for:

1) the best carrier polymer with which we could conjugate our epitope of choice
2) use of fluorescein as the hapten (or model epitope), and its conjugation to a variety of carrier polymers
3) careful fractionation and characterization of the hapten-polymer conjugates
4) determination of the optimal molecular parameters of any hapten-conjugated molecule to best induce immunogenicity.

Ultimately, the epitope of choice was to be a relevant malarial peptide. However, we began our studies using the hapten, fluorescein as a model "epitope", since peptides are more difficult to work with, and are expensive. Table 1 lists the polymers which we examined, along with their molecular characteristics.

TABLE 1: CHARACTERISTICS OF POLYMER MOLECULES STUDIED

Polymer	Polymer Composition	Molecular Characteristics
Polyacrylamide	Synthetic polyethylene polymer	Linear homopolymer, uncharged
Ficoll	Polysaccharide synthesized from sucrose	Three-dimensional, highly cross-linked heteropolymer
Dextran	Bacterial polysaccharide of glucose units	Predominantly linear, somewhat branched homopolymer
Carboxymethyl-cellulose	Carboxy-methylated plant polyglucose	Linear, negatively charged homopolymer
Polyvinyl alcohol	Synthetic polyethylene polymer	Linear homopolymer
Pneumoccal polysaccharide type 3 (PP-3)	Bacterial polysaccharide	Alternating glucose-glucuronic acid

All the polymers listed in Table 1 (with the exception of the PP-3) were haptenated with fluorescein. Size-fractions of the conjugated hapten polymers were obtained which varied in molecular mass and hapten valence and density. When the various haptenated polymer preparations were tested for immunogenicity, both *in vivo* and *in vitro*, it was found that, regardless of the chemical nature of the carrier polymer, molecules of mass greater than 100kD and with more than 20 haptens per molecule were immunogenic, while smaller molecules were not immunogenic at any of the dose ranges tested (Table 2).

TABLE 2: EFFECT OF MOLECULAR PROPERTIES ON IMMUNOGENICITY

Polymer [d]	Density (mmoles Fl/gm polymer)	Immune Response (*in vitro*)[a]	(*in vivo*)[b]
Fl_{240}Fic750	0.32	+	+
Fl_{90}Fic750	0.12	+	+
Fl_{65}Dex400	0.16	+	+
Fl_{60}Dex170	0.35	+	N.D.[c]
Fl_{95}PA300	0.32	+	N.D.
Fl_{230}PA400	0.58	+	N.D.
Fl_{160}CMC520	0.32	+	+
Fl_{26}CMC110	0.24	+	+
Fl_{110}PVA400	0.28	+	N.D.
Fl_{55}PVA200	0.28	+	+
Fl_{14}Fic40	0.35	–	–
Fl_{6}Fic35	0.17	–	–
Fl_{14}Dex40	0.35	–	N.D.
Fl_{47}PA80	0.59	–	N.D.
Fl_{6}CMC27	0.22	–	–
Fl_{14}PVA50	0.28	–	–

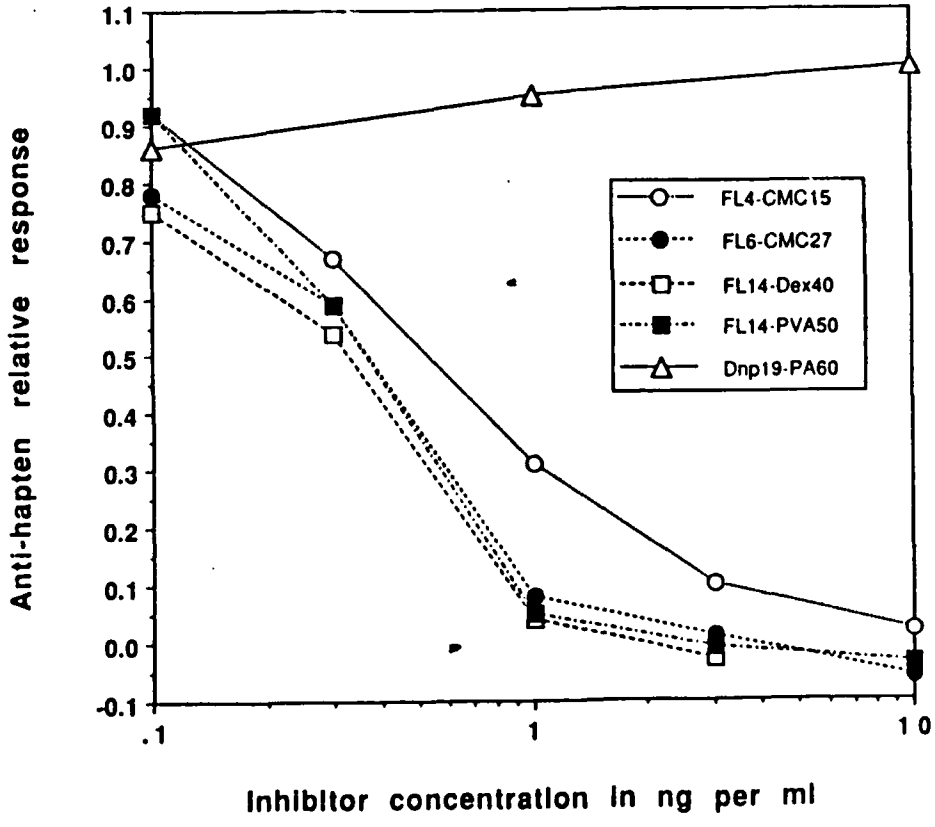
a) Determined by measuring direct anti-Fl PFC after 3 day culture of splenocytes with antigen.
b) Determined by measuring direct anti-Fl PFC of splenocytes harvested 4 days after i.p. injection of antigen in saline without adjuvant.
c) N.D.= not determined
d)Symbols used in describing polymers are illustrated in the example below:

Fl_{65}Dex400 = Hapten $_{\text{hapten valence}}$ Polymer M.W. (kD)

Dose-response curves of all immunogenic molecules (those above the dotted line in Table 2), were bell-shaped, indicating that immunogenicity increased with increasing dose up to a maximum, and then decreased with increasing dose. Importantly, smaller, densely fluoresceinated molecules with one type of carrier polymer could inhibit the response to fluoresceinated immunogenic molecules with another type of carrier molecule.

Figure 1 shows a representative example of such inhibition. Naive splenocytes were incubated with a series of solutions formulated to contain increasing concentrations of the nonimmunogenic polymers together with a constant concentration of the immunogenic polymer, $Fl_{90}Fic750$. As can be seen, the inhibitory ability of the nonimmunogenic polymers increases with increasing concentration until complete inhibition of the response to the immunogenic polymer is reached. The irrelevant hapten , Dnp, on a polyacrylamide carrier could not inhibit the anti-Fl response. This finding exhibits the phenomenon of "cross-inhibition", whereby Fl on the backbone carriers, PVA, Dex, or CMC can inhibit the anti-Fl response stimulated by Fl-Fic. This again confirmed our observation that the chemical nature of the carrier had relatively little influence on the immunological characteristics of the conjugated molecule.

FIGURE 1: Inhibition of the *In Vitro* Response to $Fl_{90}Fic750$ by Nonimmunogenic Fl-Polymers



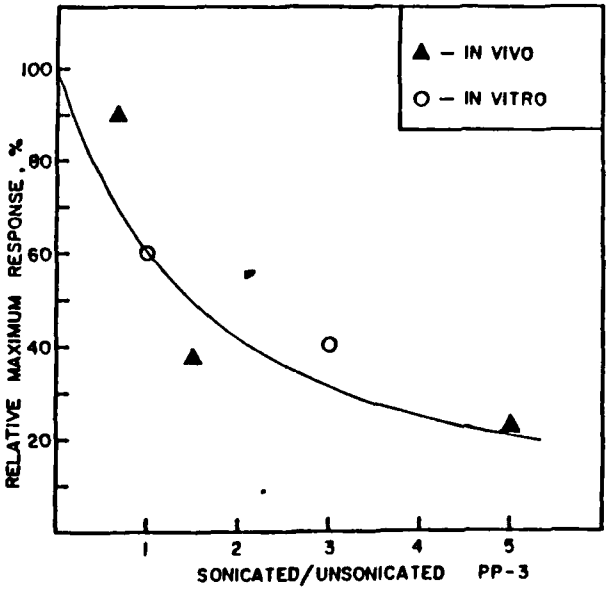
Unhaptenated preparations of PP-3 were also size-fractionated and samples of defined molecular mass obtained. Smaller molecules of less than 70kD M.W., were generated by sonication of a preparation of PP-3 with a M.W. range of 250 to 500 kD. The immunogenic parameters for these molecules were the same as those for haptenated polymers, i.e. molecules with mass greater than 100kD were immunogenic and smaller molecules, although not immunogenic, could inhibit the response to the immunogenic ones (Table 3 and Figure2).

TABLE 3: IMMUNOGENICITY OF M.W. FRACTIONS OF PP-3

PP-3 M.W. (kD)	Relative Immunogenicity *In Vitro* [a]	Immunogenic? *In Vivo*
70	0.06	barely
120	0.37	yes
220	0.84	yes
350	0.85	yes
620	1.00	yes
250-500 (unsonicated)	0.85	yes
35 (sonicated)	0.00	no

a)Immunogenicity of the 620 kD fraction at optimal dose was assigned a value of 1.00. Optimal dose response to other fractions is expressed as a fraction of that response. Immune response was determined by measuring direct IgM PFC /10^6 splenocytes.

FIGURE 2: Inhibition of Response to Unsonicated PP-3 by Sonicated PP-3



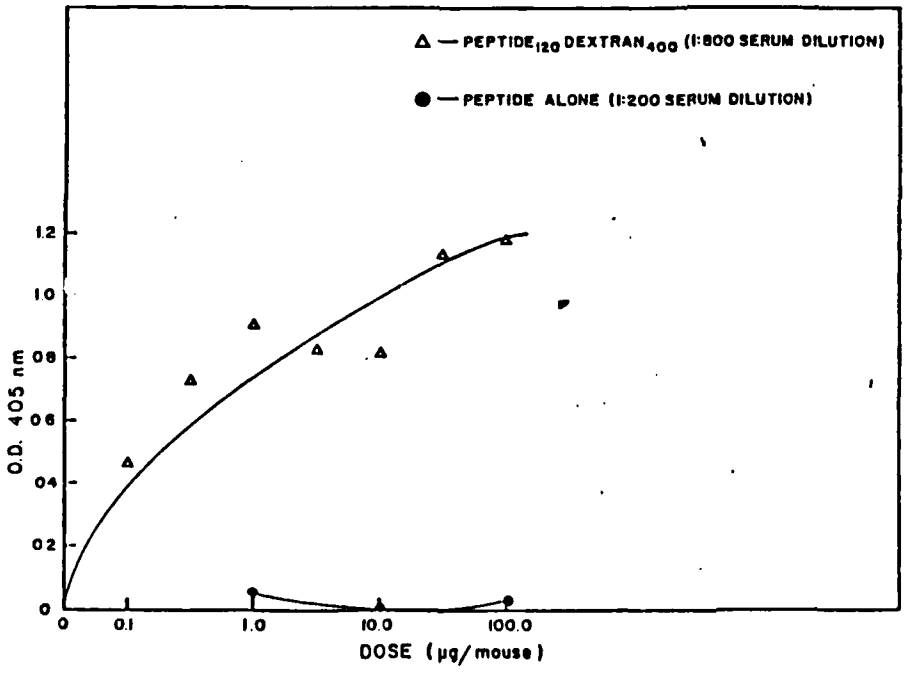
Together, the information gained from studies with both the haptenated polymers and with PP-3 indicated two important facts relevant to vaccine preparation using polymer carriers:

1) It is necessary to remove lower M.W. contaminants from polymer vaccine preparations; these contaminants are not only non-immunogenic, but are decidedly inhibitory.
2) The immune response to these types of molecules is relatively independent of the nature of the carrier molecule.

The final phase of the first year of this research project was devoted to preliminary experiments on the synthesis and immunological characteristics of a "first-try" vaccine containing a synthetic P.falciparum CS peptide linked to the carrier polymer, dextran. We chose dextran as the best carrier to use, since, as we had found, the immune response to conjugates was relatively independent of the nature of the carrier. Secondly, and of equal importance, dextran is non-toxic and has been approved for use in humans.

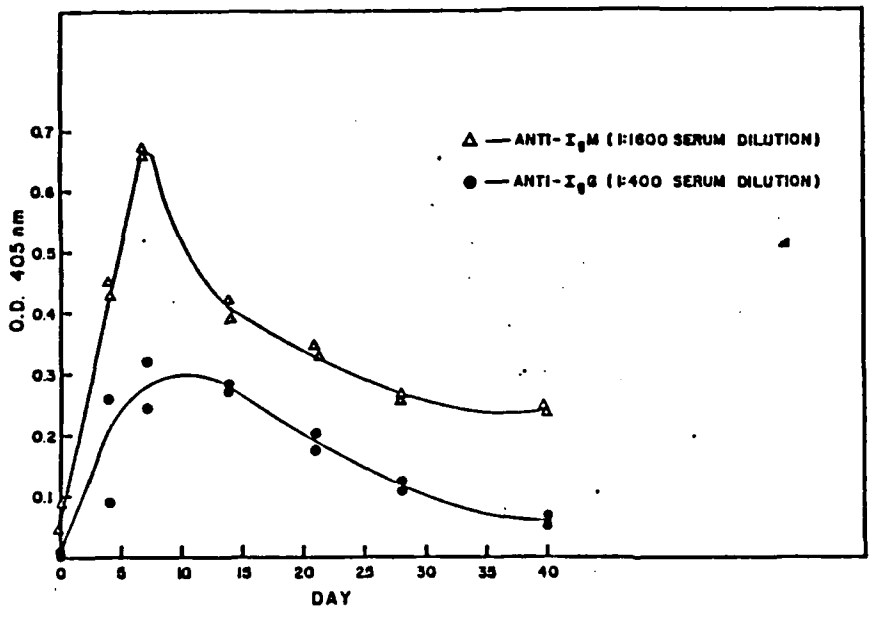
A peptide containing a triple repeat of the tetrapeptide, Pro-Asn-Ala-Asn, from the CS protein of P. falciparum was synthesized for us at Johns Hopkins Medical School. This was then coupled to several fractionated amino dextrans of different molecular weights to yield polymers with high epitope valence. The coupling procedure has been described in detail in our first year report. The conjugates were then injected i.p. into mice, in saline, in varying doses. As was predicted by us, based on the molecular characteristics of these polymers, they were found to be decidedly immunogenic, and dose-response curves were obtained indicating that the antibody response reached a plateau at 10 µg/mouse. Figure 3 shows an *in vivo* dose-response curve to peptide alone and to peptide-dextran conjugate as measured by ELISA assay of anti-peptide IgM in mouse serum 6 days after injection in saline.

FIGURE 3: Six Day Anti-Malarial Peptide IgM Response



The peptide-dextran conjugate brought up both IgM and IgG isotypes after a single injection.. Both classes of antibodies persisted for at least six weeks (Figure 4).

FIGURE 4: Time Response to 10 μg Peptide$_{120}$Dextran400



These preliminary studies confirmed our predictions that multiple copies of a peptide attached to a polymer backbone will generate an immunological response to the peptide. The response was generated in mice, without the use of adjuvants. The peptide-polymer was synthesized so as to conform to previously defined molecular parameters for immunogenicity. The tetrameric epitope from the CS protein of P. falciparum was chosen because it had already been reported to be immunogenic when coupled to protein and injected with adjuvant (Young, J.F. et al. *Science* 228, 958 (1984). The peptide-linked dextran was synthesized from a 400 kD dextran to which was attached 120 copies of the peptide. When injected in saline, i.p., into mice, this conjugate generated anti-peptide antibodies of the IgG and IgM isotypes which persisted for many weeks.

Conclusions Resulting from Year 1 Studies

We extended our studies on haptenated polymers to more precisely define those molecular characteristics which cause a molecule to become maximally immunogenic.

1) A variety of haptenated synthetic and natural polymers all require molecules of > 100kD and >20 haptens per molecule to generate an immunologic response to the hapten.
2) Even non-haptenated pneumococcal polysaccharide type3 (PP-3) must be >100kD to generate an anti-pneumococcal polysaccharide response.
3) PP-3 molecules of M.W.< 100kD can inhibit the response to larger molecules of PP-3. It is therefore important to remove smaller, non-immunogenic and inhibitory molecules from vaccine preparations.
4) Anti-hapten response to the hapten-polymer conjugate is relatively independent of the nature of the polymer carrier. Dextran was therefore chosen as the carrier of choice.
5) The above studies confirmed our prediction that multiple copies of a peptide attached to a polymer carrier will generate an immunological response to the peptide.
6) Peptide-polymer conjugates which satisfied our inferred molecular requirements for immunogenicity were synthesized, using a repeating tetrameric epitope from the CS protein of P .falciparum. The conjugate contained 120 copies of the peptide on a dextran carrier of M.W. 400kD.
7) When injected into mice,i.p., in saline, the peptide-dextran conjugate generated anti-peptide antibodies (IgM and IgG) of considerable titer, which persisted for many weeks.

Year 2 Outline:
Detailed Studies of Malarial Peptide-Dextran Conjugates

I. Choice of Peptide for Peptide-Polymer Conjugate

A. P. falciparum circumsporozoite protein peptide
B. P. bergei circumsporozoite protein peptide

II. Modification of Peptide

A. Addition of d-tyrosine to reduce exopeptidase degradation
B. Modification to allow for spectrophotometric measurement of peptide-polymer conjugate.
C. Modification of peptide to allow for possibility of labelling with ^{125}I.

III. Choice of Dextran as Optimal Polymer Carrier, and Dextran Preparation

A. Dextran is FDA approved for human use and is non-toxic.
B. Dextran size-fractionation.
C. Construction of flexible side-arms to serve as anchor points for peptide attachment.

IV. Coupling of Peptide to Dextran

A. Use of maleimide or bromoacetate groups for dextran coupling.
B. Addition of sulfhydryl groups to amino ends of peptides.

V. Immunogenicity of Dextran-Peptide Preparations

A. Kinetics of response.
B. Specificity of response.
C. Time dependence of response.
D. Effect on response of peptide density per polymer molecule.
E. Effect of dextran carrier size on response.

VI. Effect of Genetic Background on Response

A. All mouse strains tested responded to the dextran-peptide conjugates.

Aim and Scope of Year 2 Studies

The encouraging results at the end of the first year study indicated that multivalent arrays of peptides on a polymer backbone were indeed immunogenic, and could bring up anti-peptide IgG as well as IgM which persisted for many weeks. The second year, therefore, was devoted to:

1) Optimization of the methods of synthesis of malarial-peptide polymer conjugates

2) Study of the immunological characteristics of the resultant conjugates
   a) Kinetics of response
   b) Specificity of response
   c) Time dependence of response
   d) Effect on response of peptide density per polymer molecule
   e) Effect of dextran carrier size on response

3) Study of the effect of genetic background on response

Our aim in this section of the study was to investigate and to optimize the immunogenicity of selected malarial peptides conjugated multivalently to a carrier polymer. The peptides selected were derived from the known structure of the coat proteins of malaria sporozoites. We felt that we had a free choice of carrier polymers, since our previous studies had shown that the primary IgM immune response to an epitope on a polymer carrier is relatively independent of the chemical nature of the polymer carrier. Therefore, for the carrier polymer, we chose dextran, a polymer which should be readily accepted for use in human vaccines. Dextran solutions have been widely used as an emergency plasma substitute in humans for several decades. They have been found to be nontoxic, even when administered in large amounts. In addition, our previous studies with haptenated polymers indicated that of all the polymers tested, haptenated dextran stimulated the greatest actual anti-hapten response.

We chose two malarial CS peptides to conjugate to dextran- one from P. falciparum and one from P. bergei. For convenience of reference in this report, they will be referred to as Fal-pep 1 and Ber-pep 1. They were synthesized to contain the following amino acid sequences:

Fal-pep 1: A-N-P-N-A-N-P-N-A-N-P-N-A-dT-A

Ber-pep 1: G-N-P-N-P-N-P-N-R-P-N-P-N-P-N-P-dT-A

where dT refers to the d-form of tyrosine. D-tyrosine, which is not a natural constituent of each peptide, was included for two reasons. First, the presence of the d-form should reduce the rate of possible exopeptidase degradation from the free carboxyl end of the peptide. Secondly, the presence of the tyrosine would allow spectrophotometric measurement, as well as the possibility of labelling with ^{125}I.

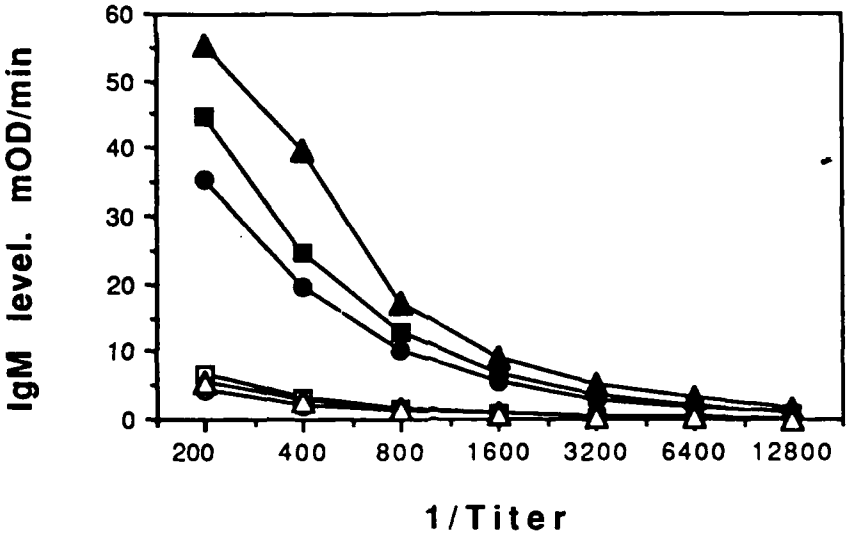
For the polymer carrier, commercial dextran preparations were size fractionated on appropriate gel filtration columns to yield material of relatively narrow molecular weight distribution. The dextran fractions were then reacted with chloroacetate under strongly alkaline conditions, to yield a series of preparations of varying degrees of covalent substitution with carboxyl groups. The carboxyl groups were then coupled to ethylenediamine through stable amide bonds, to form flexible sidearms terminated with primary amino groups, which in turn later served as the anchor points for the subsequent attachment of peptides.

Most of the coupling was done by the use of either maleimide or bromoacetate groups coupled to the dextran. These served as reactive groups for later coupling to sulfhydryl groups on the amino end of the peptide. A generally successful technique for adding the sulfhydryl groups to the amino ends of the peptides involved the use of the reagent N-succinimidyl-S-acetylthioacetate to add blocked sulfhydryl groups, which were then de-protected just prior to use in coupling the peptides.

In order to determine the immunogenicity of the peptide-dextran conjugates, saline solutions of them were injected into mice intraperitoneally. The mice were bled at various later times and the amount of antibody in their blood specifically directed against the peptide was determined by standard solid phase ELISA assay.

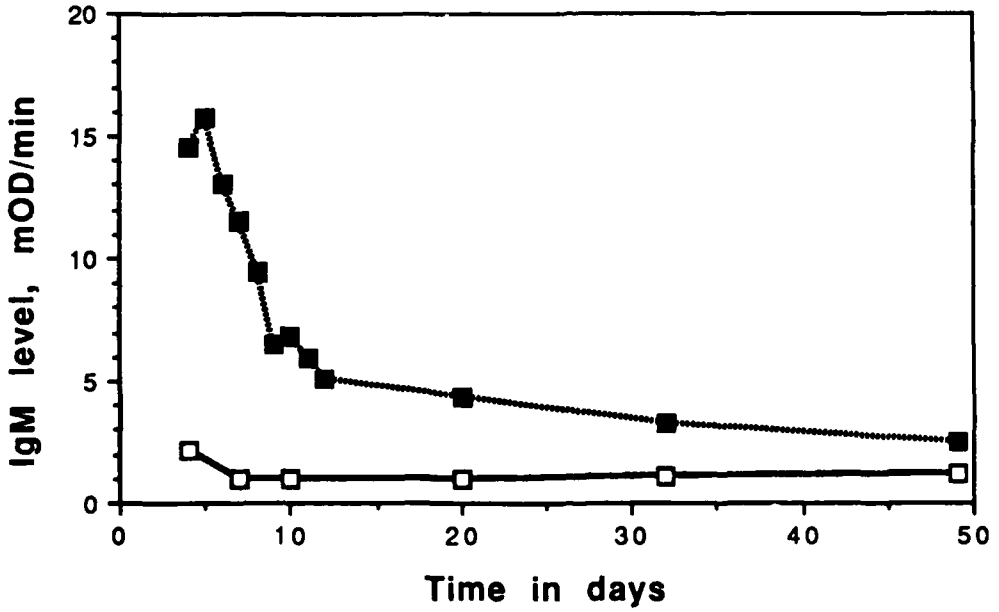
An example of the results obtained is shown in Figure 1, where the antibody titers at various dilutions of serum were measured for three individual mice injected with 1 μg Ber-pep 1 coupled to high molecular weight(1000kD) dextran. Also shown (hollow symbols) are the comparable results for mice injected with saline alone.

FIGURE 5: Anti-Peptide Antibody Response to Peptide-Dextran Conjugate



In Figure 6 it may be seen that the serum level of IgM peaks in about 6 days but remains easily measurable for many weeks after a single injection in saline without adjuvant. The filled squares represent the average anti-Fal-pep 1 IgM antibody responses of a group of three CAF_1/J mice injected with 10 μg of Fal-pep 1 coupled to dextran of molecular weight approximately one million at an epitope density of 45 peptides per 100 kD of dextran. The empty squares represent the corresponding results from a group of control mice injected with saline. The sera were diluted 800 fold for assay.

FIGURE 6: Kinetics of an Anti-Peptide IgM Response



The six day peak in response, followed by a fall over the next 40 days was observed at all doses of antigen used (from 0.1 to 100 µg peptide/ mouse).

We found a very substantial dependence of the immune response on the amount of peptide coupled to a constant amount of dextran, the so-called "epitope density". The serum IgM response, measured at day 6, is low at low epitope density, rises steeply in the range of 20 to 30 copies of peptide coupled per 100 kD of dextran, and then plateaus at higher levels of substitution of the peptide on the dextran (Fig.7).

This data suggests that a peptide epitope density of 40 to 60 copies per 100 kD of dextran may be in the optimal range for maximum effectiveness per amount of peptide injected. For the 400 kD dextran used in Figure 7, this would amount to 160 to 240 copies of the peptide per 400 kD of dextran carrier. The anti-Ber-pep 1 IgM antibody response at 6 days is shown after mice received two different doses of injected peptide, 1µg and 10µg. Serum was diluted 800 fold.

FIGURE 7: Effect of Epitope Density on Response

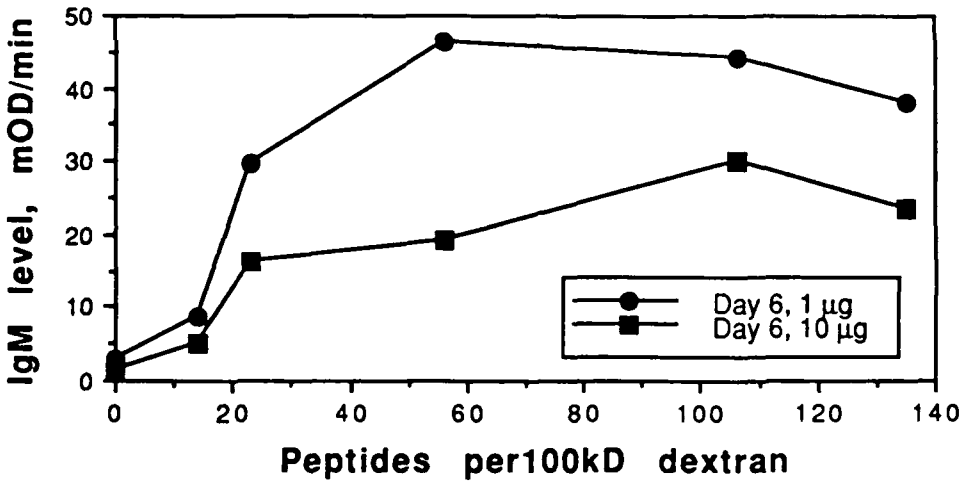
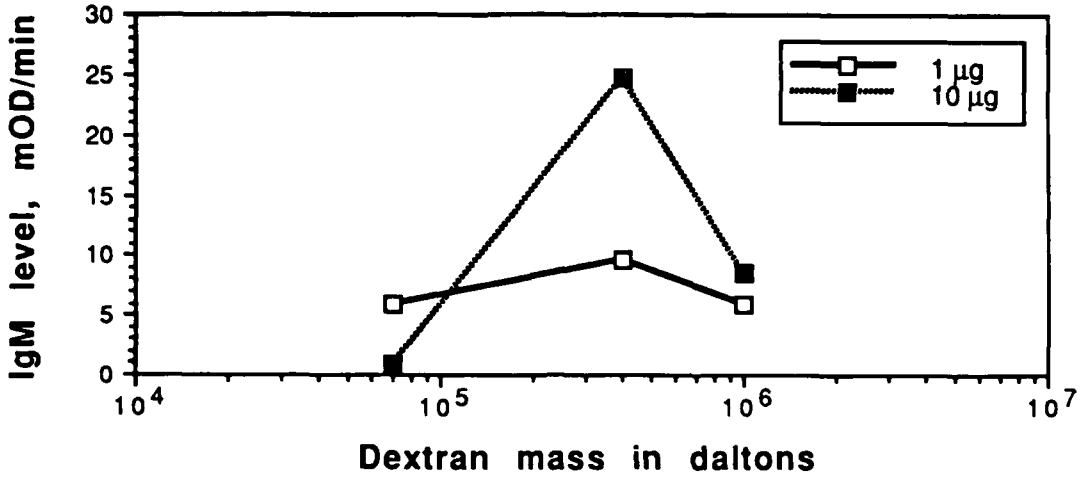


Figure 7 also demonstrates that a very substantial IgM serum response against the P. bergei peptide coupled to dextran and administered in saline without adjuvant can be generated at short times(day 6) to a small amount of peptide. The response was still substantial even after 77 days, and the slopes of subsequent curves obtained after further experiments indicated that a response lasting many more months was likely.

The variation of the immunogenicity of peptide-dextran complexes with the molecular mass of the dextran carrier is an important consideration in the design of molecules which can give an optimal immune response. To study this variable, peptide-dextran compounds containing approximately 45 copies of Ber-pep 1 peptide per 100 kD dextran (range 38 -56 peptides per 100 kD dextran) were prepared from roughly fractionated dextran preparations of molecular weights 70 kD. 400 kD, and 1,000 kD.

As may be seen in Figure 8, the peak immune response against the Ber-pep 1peptide occurred when the intermediate size of dextran was used as carrier. This indicates that dextran molecules of approximately 400 kD are near optimum size as carriers for the Ber-pep 1 peptide. As can be seen from the figure, both dose and size of carrier influence the magnitude of the antibody response. The response was measured 6 days after injection of the conjugate in saline. The molecular weights and peptide valence for the three peptides were as follows: $Pep_{27}Dex70$, $Pep_{224}Dex400$, $Pep_{450}Dex1000$, (Symbols= Peptide $_{\text{peptide valence}}$ Polymer M.W. (kD)). To determine more precisely the optimum dextran size, it will be necessarily to measure dose response curves for each size of dextran and compare the magnitude of the peak response dose for each size of dextran.

FIGURE 8: Effect of Dextran Mass on Immunogenicity

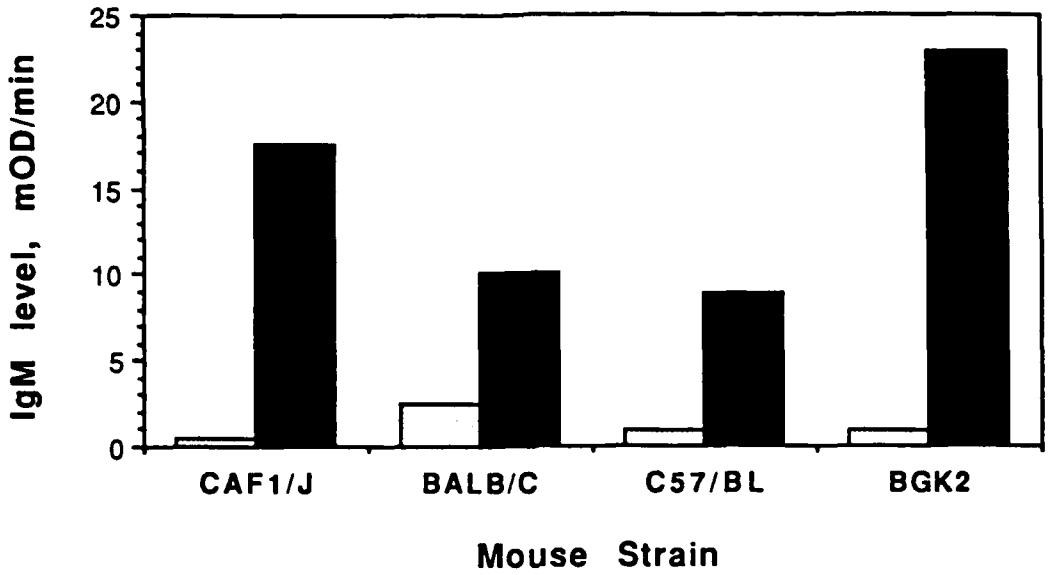


The last factor influencing response to peptide-polymer conjugates to be studied in this second year of the project was the effect of genetic background on the immune response. This study was undertaken because genetic backgrounds, particularly the MHC characteristics, of different strains of mice have been found to contribute very significantly to the level of the immune response to a number of types of antigenic material. In fact some investigators have reported that some strains of mice respond to peptides derived from the amino acid sequence of P. falciparum represented by Fal-pep 1, whereas other strains of mice do not respond.

To test whether our complexes of Fal-pep 1 with dextran brought up comparable responses in a number of mouse strains, we picked two "responder" strains and two "non-responder" strains to compare. BALB/c and CAF_1 are reported to be "responder" mouse strains which are capable of generating T cell responses to the peptide, while BGK2 and C57BL are reported to be "non-responders" which are incapable of such T-cell response.

These four strains of mice, all from Jackson Labs, were compared for their responsiveness to the same Fal-pep 1 complex as shown in Figure 9. The serum IgM anti-Fal-pep 1 response was measured 6 days after injection of Fal-pep 1-dextran complex, containing 10 µg peptide. The dextran carrier was 1000 kD mass and had 23 peptides coupled per 100 kD dextran. The lightly shaded bars indicate the background response to PBS and the darker bars indicate the response to the peptide-dextran complex. The data represent the average of values from 3 mice.

FIGURE 9: "Responders" and "Non-Responders" Make an Anti-Peptide Response



The results in Figure 9 do not indicate a substantial difference in the IgM antibody levels generated by this peptide-dextran complex for the "responder" and the "non-responder" pairs of mouse strains. If this result were to be substantiated with more peptide dextran complexes, it would have important implications for the possible usefulness of peptide-dextran complexes as vaccines for genetically outbred populations.

Conclusions Resulting from Year 2 Studies:

We made appreciable progress in synthesizing immunogenic conjugates of selected malarial peptides on a dextran polymer backbone. The immunologic characteristics of the conjugates were found to be as follows:

1) For optimal immunogenicity, the molecular mass of the dextran carrier polymer should be in the range of 400kD.
2) For optimal immunogenicity, the peptide density should be in the range of 40 to 60 copies per 100 kD of polymer.
3) The conjugates are immunogenic when administered i.p., in saline, without the use of adjuvants.
4) After injection of the conjugates, serum IgM levels reach peak levels in six days and remain easily measurable for over two months. From the slopes of the curves of IgM levels plotted against time, it is likely that the response will last many more months.
5) Although some mouse strains have been reported to be "non-responders" to some P.falciparum peptides, we find no substantial differences in the anti-peptide responses of "responder" and "non-responder" mice after injection with our peptide-dextran conjugates. This yields preliminary evidence that similar complexes might prove useful in genetically outbred populations, such as human populations.

Year 3 Outline:
Use of Polyproteins as Backbone Carriers in Peptide-Polymer Conjugates: Immunogenicity Studies

I. Polymerization of Bovine Serum Albumin (BSA) and Ovalbumin (OVA)

A. Optimization of conditions to yield water soluble and stable polymers.
B. Fractionation procedures for molecular weight determinations of the polyproteins.

II. Studies on the Immunogenicity of Polymeric BSA

A. Measurement of anti-BSA IgM; Dose response curves.
B. Effect of BSA multiplicity on the IgM response.
C. Measurement of anti-BSA IgG; Dose response curves.
D. Effect of BSA multiplicity on the IgG response.
E. Effect of multiple injection protocol vs single injection protocol on IgG response.
F. Relationship of immunogenicity to timing of injections; superiority of biweekly injection protocols.
G. Effectiveness of low dose protocols.

III. Studies on the Immunogenicity of Polymeric OVA

A. Measurement of anti-OVA IgM; Dose response curves.
B. Measurement of anti-OVA IgG; Dose response curves.
C. Effect of OVA multiplicity on the IgG response.

IV. Comparison of Poly-BSA and Poly-OVA Responses

V. Preliminary Studies on the Immunogenicity of Epitope-Polyprotein Conjugates

Aim and Scope of Year 3 Studies

For a model malarial vaccine, we had conjugated relevant peptides from P.falciparum and P. bergei to dextran. The first two years of this project were involved in optimizing those molecular properties of such peptide-polymer conjugates which would result in maximal immunogenicity in mice. We succeeded in identifying the optimal peptide epitope density and the optimal molecular weight of the dextran polymer which resulted in the stimulation of a long-lasting and sizeable anti-peptide IgM response.

Because protective, neutralizing antibodies generally tend to be of IgG isotypes of higher affinity, the third year of this project has been devoted to identifying those molecular parameters and properties which would result in optimal anti-peptide IgG antibody responses. To do this, we carried out the following:

1) Synthesis of protein polymers of defined multiplicity to replace dextran polymers as carriers.
2) Studies aimed at addressing the problem of the optimal design of protein polymers as immunogens themselves.
3) Injection protocols and doses of poly-proteins which yield maximal anti-protein IgG responses.
4) Use of poly-proteins as carriers for relevant epitopes; immunological characteristics.

Epitopes contained in, or attached to, protein molecules and administered together with adjuvants are known to be quite effective in raising substantial levels of antibody responses, some of which have been demonstrated to be effective as vaccines. The use of protein molecules as "carriers" for epitopes has a possible advantage since it can lead to substantial isotype "class switching". This often results in the production of high levels of IgG antibodies of greater affinity for the epitope than is commonly found with the IgM antibodies resulting from the use of simple polymers as carriers.

A large number of studies have shown that polymerized protein molecules can be quite immunogenic, even when administered in the absence of adjuvants. However, there is little quantitative information to define the molecular characteristics required for the optimum immunogenicity of polymerized proteins in the absence of adjuvants. The studies carried out during the third year contract period were largely aimed at addressing the problem of the optimal design of protein polymers as immunogens themselves, and also as carriers for covalently coupled epitopes, such as peptides. which could be used as components for effective vaccines.

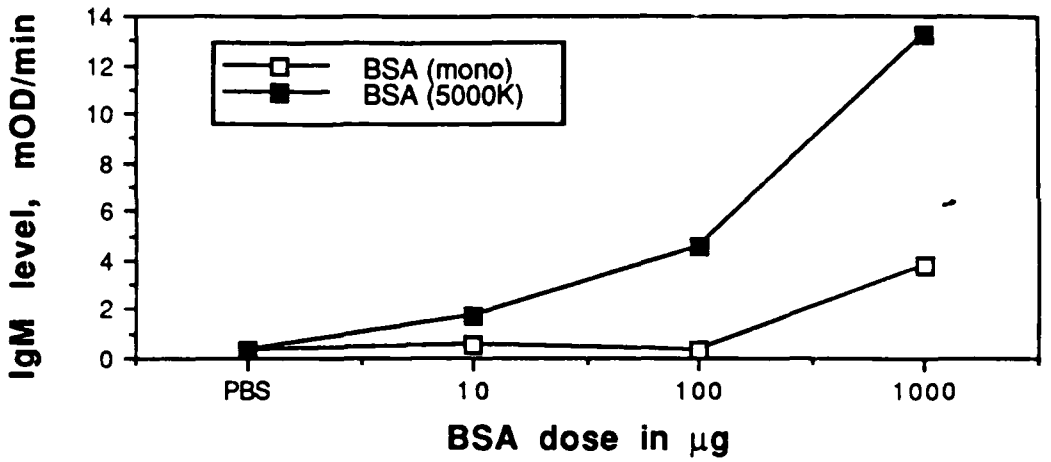
As model proteins for these studies we used two proteins, bovine serum albumin (BSA) and hen egg ovalbumin (OVA). Both of these proteins are readily available in pure form and are quite water soluble, relatively stable and quite immunogenic in mice. Conditions were found that allowed the polymerization of either BSA or of OVA to give polymers, in substantial yields, ranging from dimers to very high polymers, all of which were water soluble and time stable.

The protein monomers, oligomers and polymers, which were produced, were subjected to fractionation and repeated re-fractionation on a series of gel filtration columns until they demonstrated narrow molecular weight distributions, as measured by HPLC analysis. The molecular weight for each fraction was then determined by the use of the Model E analytical ultracentrifuge under equilibrium conditions.

We ended up with a series of preparations, each of which contained a relatively narrow range of molecular sizes of substantial time stability. These water soluble preparations were then injected into mice intra-peritoneally, without the use of any adjuvant, in order to determine their relative immunogenicity. The level of immune response was determined by measuring serum IgM or IgG antibody levels against BSA or OVA by standard solid state ELISA technique.

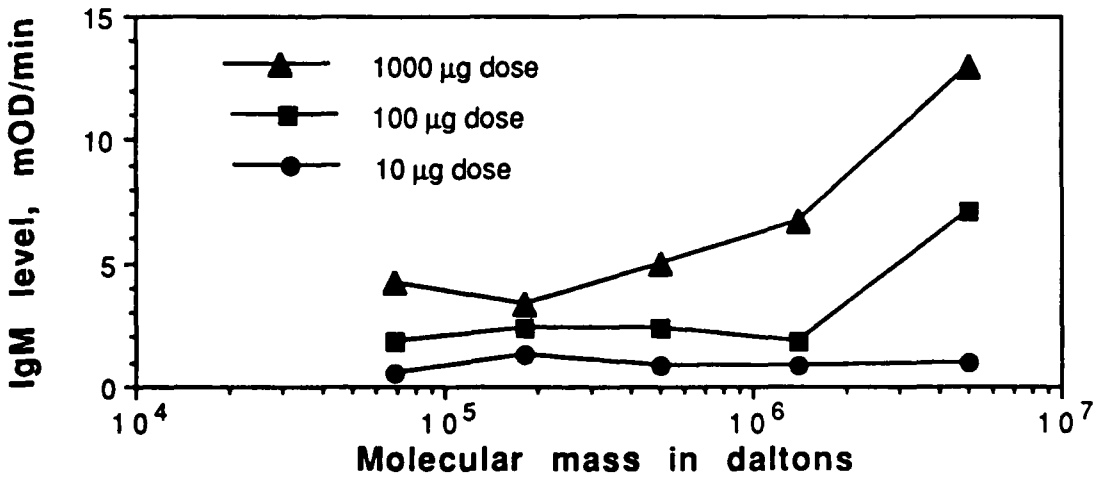
As was the case with the previous haptenated dextran studies, the anti-BSA IgM serum levels were found to rise rapidly, peaking at about 6 days and then declining to a plateau level. Figure 10 shows that soluble highly polymerized BSA (a "70-mer", containing 70 BSA monomers) is capable of raising IgM antibodies even at very low doses, whereas monomeric BSA requires substantially higher doses to bring up detectable IgM levels against BSA. The antibody response was measured at day 6 by ELISA for serum dilutions of 200 fold. The response to the injection of PBS alone is given for reference.

FIGURE 10: Dose-Response Curve of Polymerized BSA



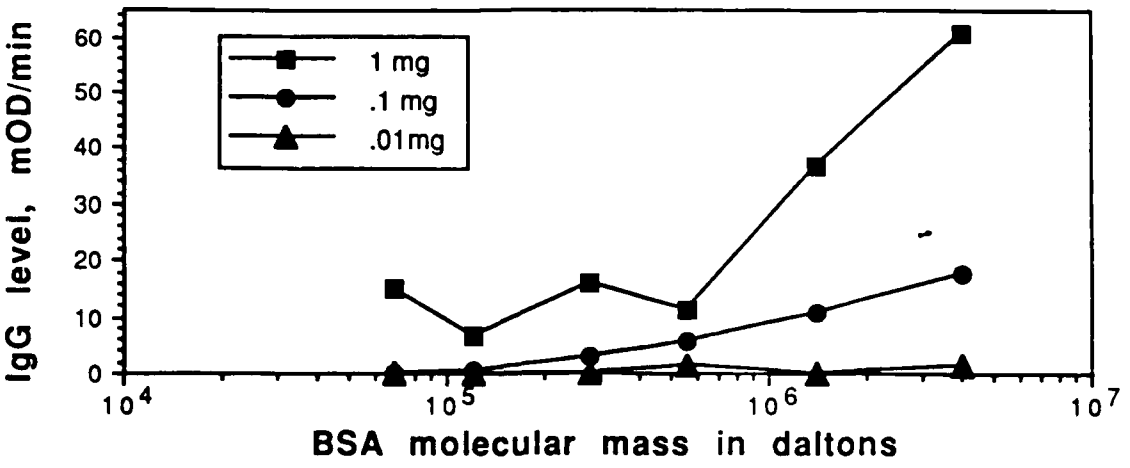
When data from a series of polymers of BSA of differing molecular weights was compared, in Figure 11, the immunogenicity, as measured by IgM levels at day 6, was found to increase most rapidly at the higher molecular weights, but was strongly dose dependent at all molecular weights. The BSA complexes contained approximately 1, 3, 7, 20, and 70 monomers per polyprotein complex. (BSA monomer has a mass of 68 kD).

FIGURE 11: Effect of BSA Multiplicity on IgM Response



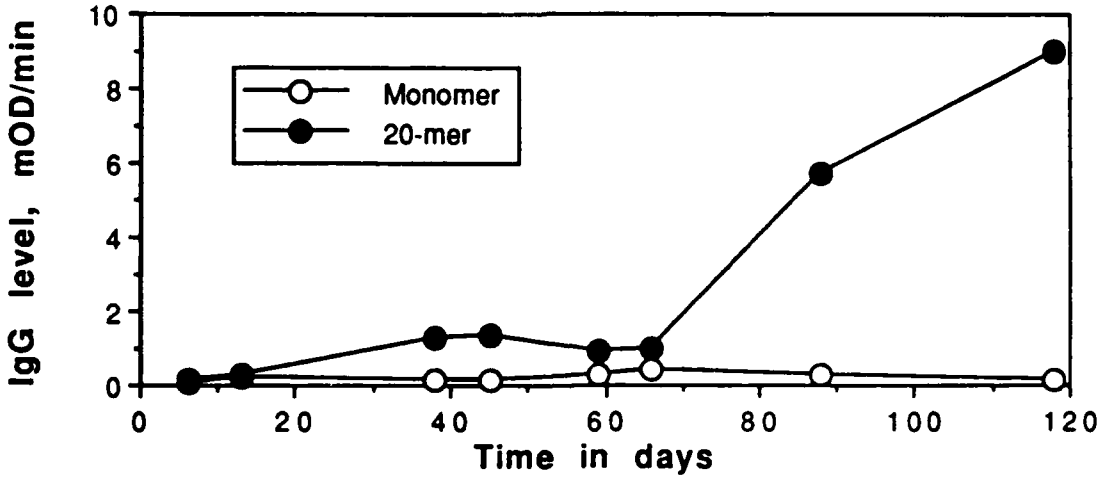
By day 14 after the injection of the soluble polymers of BSA, substantial isotypic class switching had occurred and anti-BSA IgG antibodies were found to be present for some combinations of antigen dose and molecular mass, see Figure 12. Serum was diluted 1000 fold for assay.

FIGURE 12: Effect of BSA Multiplicity on IgG Response



When multiple small injections of BSA polymers were given, the response was very dependent upon the molecular weight and dose of the BSA polymer. Even repeated injections in saline, at doses up to 100 µg, were not very effective in producing an anti-BSA IgG response, unless the preparations contained polymers of substantial size. This observation was confirmed even when the total number of consecutive injections, on a monthly basis, was increased to five, as is illustrated in Figure 13. This figure clearly shows that even very small doses (1µg) of highy polymeric protein can be immunogenic in the absence of adjuvant, if they are administered repeatedly. In this case, a total of 5 µg of poly-BSA was given.

FIGURE 13: Effect of Multiple Injections on Response to a BSA "20-mer".

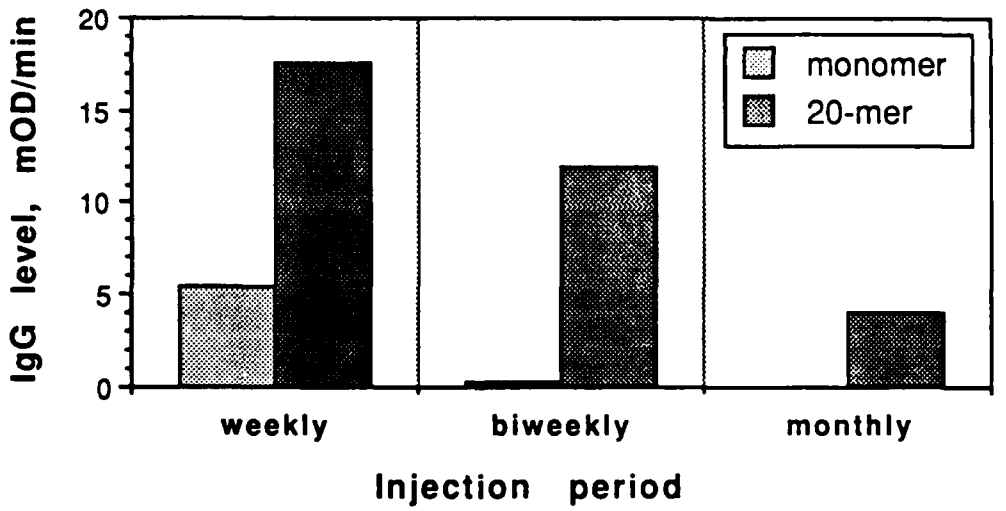


When the relationship of immunogenicity to the timing of injections was examined, some interesting facts were uncovered. The time period between successive injections of antigen was found to be very significant with respect to the level of IgG serum antibody which is produced. When a high molecular weight BSA polymer was injected into mice on a weekly, biweekly or monthly injection schedule, the immune responses differed dramatically for the different procedures.

The antibody levels rose more rapidly in mice receiving weekly injections, but the final levels did not exceed those of mice on a biweekly protocol. When compared on the basis of the total amount of protein injected, the biweekly and monthly protocols brought up approximately equal amounts of antibody by the end of the experimental period. Since the weekly protocol had reached a plateau level while the biweekly level had not, it is likely that the biweekly protocol would lead to higher antibody levels at longer times. Perhaps most interesting is the fact that the level of antibody produced by the administration of the BSA polymer in saline approached the level of antibody produced by the use of Freund's adjuvant, which is normally considered to be the "gold standard adjuvant" in animals.

The data just described resulted from the repeated injection of relatively large doses, 100 μg, of polymerized BSA. In an additional experiment to test the effect of lower dose injection protocol, mice were injected repeatedly with 1 μg doses, in saline, of either monomeric BSA or 1400 kD polymerized BSA (20-mer). Injections were made on a weekly, biweekly or monthly schedule until a total of 5 μg had been injected. The mice were bled 6 days after the last injection,and the serum was diluted 1000 fold, giving the results shown in Figure 14

FIGURE 14: Effects of Low Dose Protocols



For the low doses used in Figure 14, the more frequent injections of polymeric BSA gave the higher levels of antibody. With the weekly protocol, monomeric BSA induced some antibody formation, but the monomer gave only baseline levels of antibody with either biweekly or monthly injection protocols.

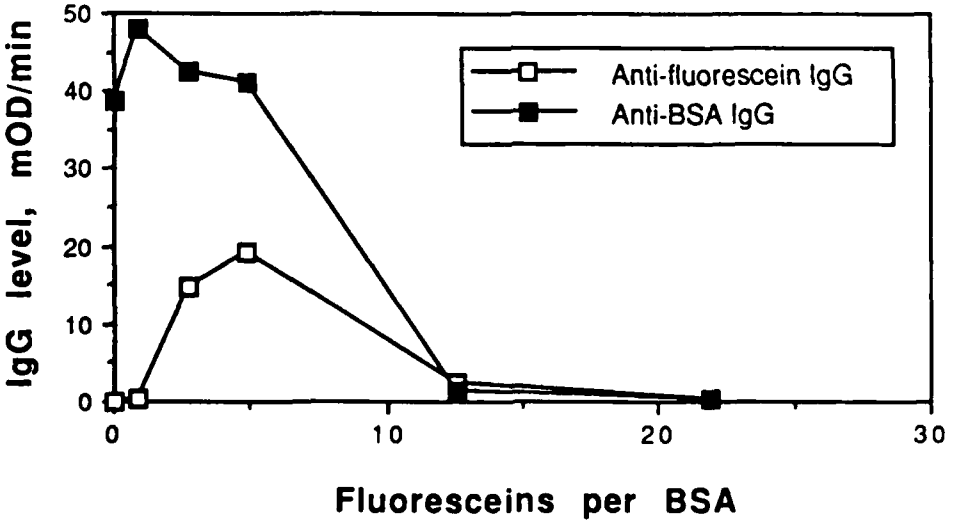
All the foregoing results applied to studies with polymerized BSA, however, results very similar to those obtained with polymerized BSA were obtained when polymerized ovalbumin, OVA, was used as antigenic material. As with poly-BSA, single saline injections of polymerized OVA, resulted in anti-OVA IgM responses, while multiple injections assayed after longer time periods, resulted in substantial IgG responses.

When glutaraldehyde polymerized OVA was size fractionated and several different doses of the individual fractions were injected three times at monthly intervals, the immune response was found to depend strongly on the OVA-polymer size and the dose. The variations of response with dose and polymer size were roughly comparable to those found with polymers of BSA.

After having optimized the immunogenicity of the polymerized protein carrier, the final phase in this research plan involved the conjugation of relevant peptides to these highly immunogenic multimeric protein carriers. As with our first year studies, we began this phase by first using haptens rather than peptides because peptides are more difficult to work with, their chemistry is more complex, and they are more expensive.

In order to determine the immune response to a hapten on polymerized protein, fluorescein was coupled to BSA polymer and the immune response was determined after several injections, Figure 15. Samples of a BSA polymer (20-mer) were haptenated with fluorescein isothiocyanate, yielding preparations with different amounts of fluorescein per BSA monomer unit. Mice were injected with 4 biweekly doses of 100 µg each in saline, for a total dose of 400 µg. Serum was assayed for IgG antibodies to fluorescein and to BSA.

FIGURE 15: Comparison of the Anti-Fluorescein and Anti-BSA Responses Generated by a Fluorescein-BSA Polymer



These preliminary results have indeed been encouraging. The anti-hapten response was of the desired IgG isotype. It rose with increasing degree of substitution, peaking at approximately 5 fluoresceins per BSA monomer unit, or a total of 100 fluoresceins per BSA 20-mer. It then fell rapidly to very low levels with increasing substitution. On the other hand, the immune response to the BSA itself remained relatively constant with increasing fluorescein substitution until approximately 5 haptens had been added, whereupon it, rather surprisingly, also fell rapidly. This suggests that there may also be an optimum level of substitution of polymerized proteins with peptide epitopes of types potentially useful for vaccines. Time has not allowed the optimization of all parameters of this promising beginning, but we anticipate that optimization of hapten, and then peptide, conjugation to these protein polymers will yield important information as to the construction of maximally immunogenic adjuvant-free vaccines using this type of chemistry.

Conclusions Resulting from Year 3 Studies:

Because protective, neutralizing antibodies generally tend to be IgG isotypes of higher affinity, the third year of this project has been devoted to identifying those molecular parameters and properties of peptide-polymer conjugates which would result in maximal IgG anti-peptide responses.

The first step has been to use poly-proteins as carrier molecules instead of dextran polymer. Because so little is known of the molecular and immunological properties of poly-proteins, considerable emphasis was placed on a study of these parameters in themselves, before attempts were made to use poly-proteins as carriers. The poly-proteins synthesized were poly-BSA and poly-OVA. The immunological characteristics elicited by these polymers in mice were as follows:

1) Polymeric BSA and OVA, administered without adjuvant, stimulate considerable IgM and IgG anti-protein responses.
2) The immunogenicity of these poly-proteins increases with increasing protein multiplicity.
3) Immunogenicity of poly-proteins is strongly dose-dependent, the immunogenicity increasing with increasing dose. 100 μg per mouse was the highest dose tested; however, (and importantly), even doses as low as 1 μg per mouse, administered in the proper protocol, gave rise to considerable anti-protein IgG antibody.
4) The time period between successive injections of poly-proteins was found to be very significant with respect to the level of IgG serum anti-protein antibody produced. A biweekly injection protocol of poly-protein in saline was most effective.
5) Polyproteins are effective carriers. Flu-Poly-BSA was synthesized as a "model" carrier-epitope conjugate.
6) Fluoresceinated -poly-BSA generated anti-fluorescein IgG responses, the response rising to a peak with increasing degree of substitution, and then falling rapidly with increasing substitution.

Summary and Future Directions

Considerable and encouraging progress has been made in fulfilling the aims set forth in our original research proposal. Using relevant peptides from the circumsporozoite coat protein of P.falciparum and P.bergei, we have successfully constructed peptide-polymer conjugates of optimal immunogenicity in mice. We chose dextran as the carrier polymer of choice after experimenting with a number of carriers of different chemical and physical characteristics. The molecular parameters crucial for the construction of the immunogen were determined. These involved using a dextran carrier molecule of optimum molecular mass coupled to peptide in such a way as to yield an optimum peptide density and valence per molecule. Importantly, as we originally proposed, these conjugates were immunogenic without the use of adjuvants. The constructed antigenic conjugates were essentially of the T-cell Independent type, and gave rise primarily to antibody of the IgM isotype.

Building on the information gained from the determination of the optimal molecular characteristics necessary for peptide-dextran conjugates to stimulate IgM responses, we next concentrated on stimulating IgG antibody responses. This was done because protective, neutralizing antibodies are often IgG isotypes of higher affinity. We synthesized the polyproteins poly-BSA and poly-OVA for use as carriers. By themselves, these polyproteins induced IgG as well as IgM responses and immunogenicity increased with increasing multiplicity of protein. Injection protocols were optimized so as to yield maximal immunogenicity using the lowest doses feasible. As with peptide-dextran conjugates, polyproteins were immunogenic without adjuvant use, and the resultant immune response approached in magnitude the level of antibody produced by injecting polyproteins in Freund's adjuvant.

Making use of the above information as to the optimal protocols and doses to be used with polyproteins, preliminary model experiments have been carried out to explore the use of polyproteins as carriers for conjugation to relevant peptides. Preliminary experiments using the hapten, fluorescein, as a "model epitope" have been carried out. Fluoresceinated poly-BSA generated anti-fluorescein IgG responses, the response rising to a peak with increasing degree of substitution, and then falling rapidly with increasing substitution, indicating that there is an optimal epitope density beyond which response diminishes.

Obviously, the next step after these encouraging results with haptenated polyproteins is to conjugate malarial peptides to polyproteins and optimize their immunogenicity. These peptide-polyproteins should be expected to fulfill many of the prerequisites for an effective stimulator of anti-malarial IgG without the use of adjuvants. The effectiveness of such a vaccine construction would ultimately depend on testing for ability to protect against malarial infection in living animals.

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